

USE OF TWO-DIMENSIONAL ELECTROPHORESIS TO IDENTIFY AND MAP NEW MOUSE GENES¹

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ABSTRACT

Cytosol polypeptides from mouse liver have been examined using two-dimensional electrophoresis. About 250 spots were readily discernible. When cytosols from strains BALB/cBy and C57BL/6By were compared, eight genetically determined differences were observed. Other strain pairs show comparable numbers of differences. These eight phenotypes were scored in seven recombinant inbred lines derived from the two parental strains, and their strain distribution patterns were compared with previously determined patterns for other genetic markers that differ between the two progenitor strains. Using this information, tentative chromosome assignments for the genes controlling five of the variant phenotypes have been made, and two of the assignments have been confirmed using congenic resistant strains. These eight genes will be useful reference markers in future crosses designed to map new genes.

TWO-dimensional electrophoresis (O'FARRELL 1975) has been used to analyze known mutants in the resolution of several genetic problems (FOX 1976; PIPERNO, HUANG and LUCK 1977; MILMAN *et al.* 1976; STEINBERG *et al.* 1977). We have used this technique to search for new genetically determined variations of polypeptides in mouse strains and to map the genetic loci involved.

When mouse liver cytosol is examined using two-dimensional electrophoresis with Coomassie brilliant blue R250 staining, about 250 polypeptide spots can be identified readily. In a recent study using mouse tissue, KLOSE and WALLENBERG-PACHALY (1976) reported 342 spots in liver supernate. If 5% of these polypeptides are genetically different between two mouse strains, we should be able to detect at least ten differences between the two strains. The ability to determine the phenotype of an individual mouse for ten separately determined characteristics using a single technique will greatly aid in mapping new mouse genes.

For this study the progenitor strains for the first set of recombinant inbred (RI) strains derived by BAILEY (1971) have been compared. RI strains are obtained by crossing two strains of mice and developing a number of inbred strains from the F₂ generation. Chromosomes from the two original parents are randomly distributed among the mice of the F₂ generation, and during the inbreeding process a different set of chromosomal regions becomes fixed in each

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line. Although in general linked genes stay together, recombination does occur. After 20 generations of inbreeding, each RI strain is essentially homozygous for a set of chromosomes drawn at random from the two original progenitor sets. The most readily available set of RI strains are the seven C × B strains developed by BAILEY (1971) between BALB/cBy (strain C) and C57BL/6By (strain B). In this study the polypeptides in liver cytosols of mice from the two parental strains have been compared, and the interstrain differences in polypeptides have been analyzed in the seven RI strains derived from these two strains.

MATERIALS AND METHODS

Sample Preparation: Mice (8 to 18 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, Maine). Excised livers were homogenized for one minute in 5 volumes of ice-cold 0.02 M imidazole (HCl) buffer, pH 7.4, with a 13-mm Polytron homogenizer (Kinematica GMBH, Lucerne). The homogenates were centrifuged at $100,000 \times g$ for one hr in the Beckman L2-65B Ultracentrifuge using either the SW50.1 or the 50Ti rotor. The supernatant solution was stored at -20° . Protein was determined using the microbiuret method (GOA 1953).

Up to 800 μ g of mouse liver cytosol protein may be loaded onto the gel without producing severe distortion of the pattern. For each mixture of proteins the maximum sample size may differ.

Each sample was prepared as follows. Urea (142 mg) was placed in a small tube. Thirty μ l of triton (10%), 15 μ l of β -mercaptoethanol (10%), 55 μ l of water and 50 μ l of sample were added, in the order listed. The urea was dissolved at room temperature and the mixture allowed to sit at room temperature for one hr before being transferred to the top of the first dimension gel.

Two dimensional electrophoresis: Two dimensional electrophoresis (isoelectric focusing in an acrylamide gel rod, followed by electrophoresis in the presence of SDS in an acrylamide slab gel) was carried out according to the methods of O'FARRELL (1975), with the following modifications. In the isoelectric focusing dimension, Triton X-100 was used in place of Nonidet P-40 and the ampholytes, which were 2% of the final gel mixture, contained 20% pH range 3 to 10, 60% pH range 5 to 7 and 20% pH range 7 to 9. The acrylamide concentration in the running gel for the second dimension was 11%. The first dimension gels were run in a Buchler disc gel apparatus, using glass tubes 20 cm long, inner diameter 3 mm, filled to a height of 12 cm. The second dimension was run in a Hoeffer vertical slab gel apparatus, using a 1.5 mm spacer, with running gel 10 cm high.

Gel processing: After the tracking dye, bromophenol blue, had reached the edge of the gel, the gels were removed, coded and stained overnight with gentle shaking with 0.25% Coomassie Brilliant Blue R 250 in methanol: water:acetic acid, 5:5:1. The gels were destained in the methanol, acetic acid, water mixture with gentle shaking for four to seven hrs. Gels were then placed in 20% methanol overnight, dried under vacuum between two layers of dialysis membrane, using a Bio-rad gel slab dryer, and analyzed. The dried gels can be kept indefinitely if first pressed with a heavy weight to counteract a tendency to curl.

Standards: After isoelectrofocusing, the pH gradient was determined by placing 2 mm sections from a duplicate gel into 0.6 ml of recently boiled water and reading the pH after two hrs. Molecular weight standards were run in the second dimension by placing a solution containing 5 μ gm of each standard in a slot made at one end of the agarose holding the disc gel to the top of the slab gel. Standards used were lysozyme, chymotrypsinogen, ovalbumin, albumin and β -galactosidase.

RESULTS

Strain comparison: The two dimensional pattern of spots obtained from strain C (BALB/cBy) mouse liver cytosol is shown in Figure 1A, and that from strain

B (C57BL/6By) in Figure 1B. These two patterns are very similar, with most spots in identical relative positions on the two gels. Spots that are present in the pattern from one strain, but not in the other, or that differ reproducibly in intensity, are indicated by arrowheads. In the case of variant polypeptides with molecular weights 20,000 to 30,000, the spots have also been numbered, for ease in identification.

Nomenclature: The known properties of the variant polypeptide spots include molecular weight, isoelectric point and organ of origin. In naming the phenotypes, one letter has been used to indicate the organ, in this case L, and two letters to indicate the decade of the polypeptide molecular weight, TN for teens, TW for twenties, TH for thirties and FO for forties. For example, one poly-

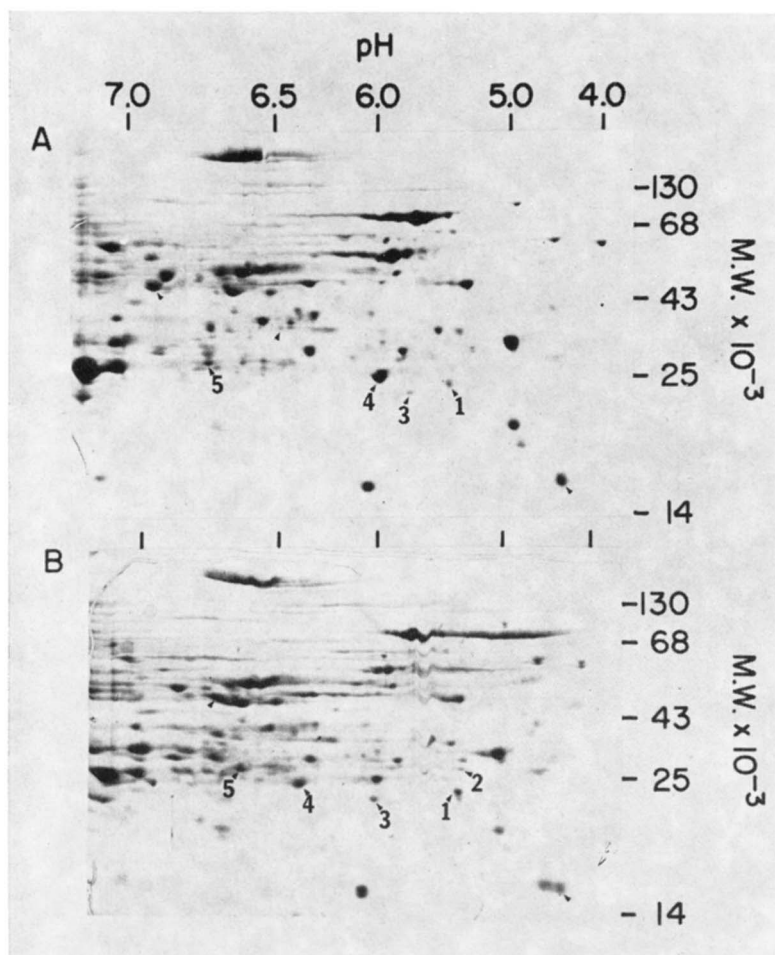


FIGURE 1.—Two dimensional electrophoresis of mouse liver cytosol polypeptides. (A) (upper) strain BALB/cBy(C). (B) (lower) strain C57BL/6By(B). Variant polypeptides are marked with an arrowhead. Polypeptides in the molecular weight range around 25,000 are also designated by a number (see paragraph on nomenclature).

peptide is designated LFO-1, indicating that it is the first polypeptide found in the 40,000 to 50,000 molecular weight range. The gene symbol is *Lfo-1*. The allele determining the variant with the more negative isoelectric point (pI) is designated *Lfo-1^a*, while the other allele is *Lfo-1^b*. The phenotypic designations are LFO-1A and LFO-1B, respectively.

This system of nomenclature has been chosen to comply with the nomenclatorial rules in the mouse (LYON 1977) and to include as much information as is currently available about the polypeptides concerned. In many cases it may represent a provisional nomenclature and be replaced by a name indicating the biochemical activity associated with the subunit.

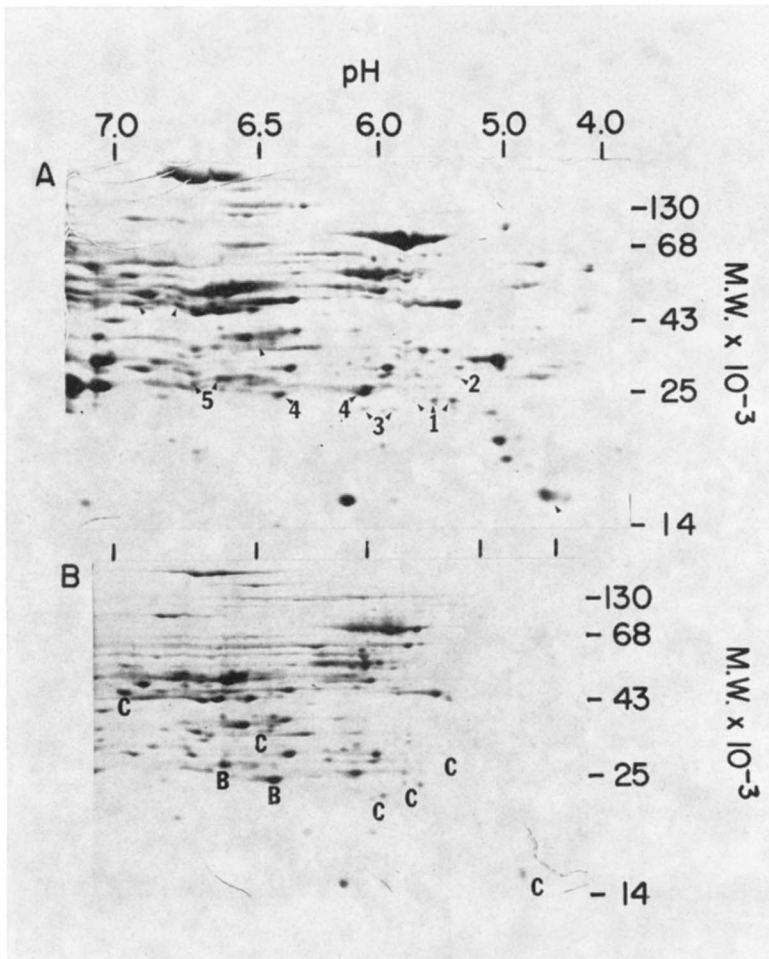


FIGURE 2.—Two dimensional electrophoresis of mouse liver cytosol polypeptides. (A) (upper); F_1 heterozygote between strains C and B. (B) (lower): recombinant inbred line $C \times BG$. In (A), variant polypeptides are indicated by arrowheads. Note that for electrophoretic variants the phenotypic forms of both parents are present. In (B), the variant polypeptides are designated either B or C, indicating the parental contribution of the phenotype.

Analysis of differences: The differences between the strains BALB/cBy and C57BL/6By are of two major types. In the case of most electrophoretic variants, heterozygotes of the F₁ generation (Figure 2A) contain pairs of spots of the same molecular weight but different pI that correspond to the individual spots in strain B and strain C. Only one of the pair of spots is present in each RI strain. The two-dimensional pattern of polypeptide spots from C × B strain G is shown on Figure 2B. The spots have been scored as either B-like or C-like in phenotype. These observations indicate that each pair of polypeptide spots represents the products of alleles at a single genetic locus or at closely linked loci. Although there are only seven C × B RI strains, the hypothesis that the pair is controlled by a single locus is the simplest hypothesis available at this time. As most of these gene pairs differ between at least one other set of RI strain progenitors, further information will be available as other RI strains are analyzed. The major exceptional variant, LTN-1, consists of three spots. The phenotypic differences are in the intensities of the spots and are described in detail in the next section.

A second set of differences between the strains can be designated quantitatively. For this type of variant, a spot is present in one strain, absent in the other and diminished in intensity in the F₁ hybrid. In addition, no companion spot with the same molecular weight but different pI can be found in the other strain. Some of these spots may be electrophoretic variants in which the second spot is masked by the density of the surrounding spots.

TABLE 1

Variant polypeptide spots and their distribution among CXB RI strains

Polypeptide spot	Phenotype* in strain		Strain distribution† pattern in RI strains							Loci with‡ identical distribution	Tentative§ chromosome assignment
	B	C	D	E	G	H	I	J	K		
LTN-1	B	A	B	C	C	C	B	C	B	<i>Mup</i>	4
LTW-1	A	B	C	C	C	B	B	B	C	<i>Mod-1</i>	9
LTW-2	A	O	B	C	C	C	C	B	C	<i>H-17</i>	—
LTW-3	B	A	C	C	C	B	B	C	C	<i>H-7</i>	9
LTW-4	B	A	C	B	B	C	C	B	C	—	—
LTW-5	A	B	C	C	B	B	C	C	B	<i>Car-1</i>	3
										<i>H-28</i>	—
										<i>If-1</i>	—
LTH-1	O	A	B	C	C	C	B	B	C	—	—
LFO-1	A	B	B	B	C	B	C	B	B	<i>Hbb</i>	7
										<i>H-1</i>	
										<i>c</i>	

* Phenotypic designations of variant spots in the parental strains (C) and (B) are given. For electrophoretic variants, (A) indicates the polypeptide with low isoelectric point. For null variants, (O) indicates the phenotype with the missing spot.

† Strain distribution patterns among the CXB RI strains, D, E, G, H, I, J and K are indicated according to which parent has the same phenotype. This facilitates comparison with strain distribution patterns of alleles at other loci.

‡ Only those loci with identical strain distribution patterns are listed. There are a number of loci whose strain distribution pattern differs in only one line from that a polypeptide spot.

§ Chromosome assignments are tentative, as with seven RI strains, even identical strain distribution patterns does not ensure linkage.

The polypeptides that have been scored and their segregation patterns among the RI strains are shown in Table 1. For each designation, at least two animals from each RI strain have been studied. Consistent scoring for each polypeptide has been found within an RI strain. Each variant polypeptide is discussed in detail in the following sections.

LTN-1: This variant has a molecular weight of approximately 17,000 and pIs from 4.3 to 4.5. The LTN-1B phenotype, found in strain B, (Figures 1B and 3) consists of a row of three spots of similar molecular weight, approximately equal intensity, and slightly different pIs. In the LTN-1A phenotype, found in strain C (Figures 1A, 2B and 3), the spot with highest pI is very intense, the middle spot is essentially missing, and the spot with lowest pI faint. Each RI strain showed one of these progenitor phenotypes. In the F₁ (Figures 2A and 3, the phenotype is intermediate between the parental phenotypes, with an intense basic spot and two lighter acidic spots.

LTN-1 probably represents the major urinary protein, MUP, that is synthesized in liver. The phenotypes expected from electrophoretic studies of MUP, and segregation of the LTN-1 phenotypes in the liver cytosols from the RI strains

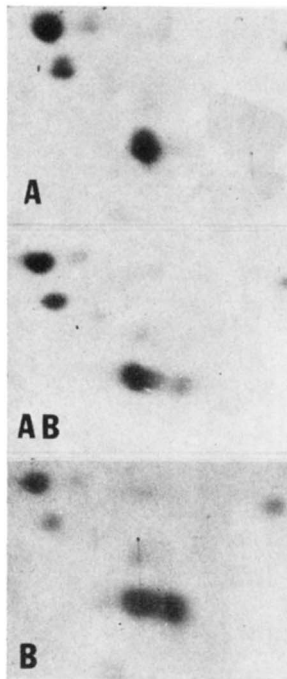


FIGURE 3.—Enlargement of part of two dimensional electrophoresis patterns showing LTN-1. In this and all subsequent figures, the top panel is obtained from progenitor strain BALB/cBy (strain C), the bottom from progenitor strain C57BL/6By (strain B), and the middle panel from the F₁ heterozygote obtained by crossing the two progenitor strains. Note that LTN-1A phenotype in strain C consists of one major basic spot, with very small amounts of the acidic spots, while the LTN-1B phenotype in strain B consists of three spots of approximately equal intensity. The LTN-1AB phenotype appears intermediate between the two homozygous phenotypes.

(Table 1) is identical with the segregation of the MUP phenotype in urine (POTTER *et al.* 1973). Thus, for LTN-1, the probable identity of the protein is known, and the gene maps on Chromosome 4 (HUDSON, FINLAYSON and POTTER 1967; FINLAYSON, HUDSON and ARMSTRONG 1969).

LTW-1: The second phenotype in Table 1, *LTW-1*, is also complex. In the heterozygous F_1 animal (Figures 2A and 4), the *LTW-1AB* phenotype consists of three spots, all about 23,000 molecular weight, but differing in pI. They are farther apart on the gel than the components of LTN-1. The *LTW-1A* phenotype, found in strain B (Figures 1B and 4), consists of the two more acidic spots, with the more basic spot very faint and the more acidic one quite intense. The *LTW-1B* phenotype, found in strain C (Figures 1A and 4), consists of the two more basic spots, again with the more acidic of the two staining more intensely. Label incorporation studies (unpublished results) show that for each phenotype the more basic spot is heavily labelled after a short pulse, while after longer

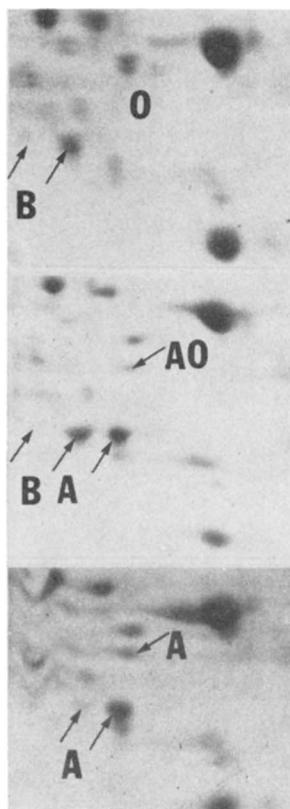


FIGURE 4.—Enlargement of part of two dimensional electrophoresis patterns showing *LTW-1* and *LTW-2*. The three panels are arranged as in Figure 3. Upward pointing arrows indicate polypeptide spots of *LTW-1*. Note the two spots in both homozygous phenotypes, and three spots in the F_1 heterozygote. Downward pointing arrows indicate the polypeptide spots of *LTW-2*. Note the presence of spot in strain B, absence in strain C, and presence of low intensity spot in the F_1 heterozygote.

pulses the label is chased into the more acidic spot. This set of polypeptides is also present in kidney supernates and is probably a major plasma component.

The two phenotypes, LTW-1A and LTW-1B, probably represent two alleles at a single locus, *Ltw-1*. The alleles of locus *Mod-1* (malic enzyme), mapped to Chromosome 9 by SHOWS, CHAPMAN and RUDDLE 1970), are distributed among the RI strains with a pattern identical to that for the alleles of locus *Ltw-1*. This suggests that *Ltw-1* also is located on Chromosome 9.

LTW-2: Polypeptide spot LTW-2 has a simple phenotype. The spot is present in strain B and absent in strain C (Figures 1 and 4). In the heterozygote, the spot is present, but less intense than in strain B. Its strain distribution pattern is identical to the pattern for histocompatibility genes *H-17*, *H-34* and *H-38*. These histocompatibility genes have not yet been given chromosome assignments.

LTW-3: The polypeptide, LTW-3, is an electrophoretic variant in which the *Ltw-3^b* allele, found in strain B (Figures 1 and 5), codes for the more basic polypeptide. The LTW-3B polypeptide coincides on the gel with another polypeptide.

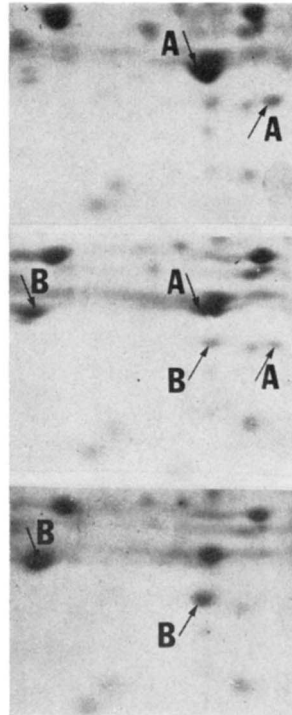


FIGURE 5.—Enlargement of part of two-dimensional electrophoresis patterns of LTW-3 and LTW-4. The three panels are arranged as in Figure 3. Upward pointing arrows indicate the polypeptide spots of LTW-3. Note that the spot marked (A) in the upper panel is missing in the lower panel, while the spot marked (B) in the lower panel is much lighter in the upper panel. In the F_1 heterozygote both (A) and (B) are present, with intermediate intensity. Downward pointing arrows indicate the polypeptide spots of LTW-4. The intense spot marked (A) in the upper panel is missing in the lower panel, where a spot marked (B) (missing in the upper panel) is seen. In the F_1 heterozygote, both spots are present.

When the more acidic polypeptide is seen in strain C, a faintly staining spot remains at the position of LTW-3B. The heterozygote shows less intense spots in both positions. The strain distribution pattern between the C \times B RI strains for *Ltw-3* is identical to that of the histocompatibility locus *H-7*, which maps on Chromosome 9 (BAILEY 1975). Thus, *Ltw-3* has been tentatively assigned to Chromosome 9, close to *H-7*.

LTW-4: The polypeptide spot, LTW-4, is one of the more prominent spots on the gel. The two phenotypic forms, LTW-4A in strain C and LTW-4B in strain B (Figures 1 and 5), are the most widely separated of all the electrophoretic variants in this study. The variant LTW-4A has the same isoelectric point as another fairly prominent spot, but migrates a little farther in the second dimension, indicating a slightly lower molecular weight. Both LTW-4A and LTW-4B are present in the heterozygote, but with reduced intensity. The strain distribution pattern for *Ltw-4* differs from any so far reported; hence it may be located in a region of the genome with few known loci.

LTW-5: The most basic of the variants in the 20,000 to 30,000 molecular weight range is LTW-5. The two phenotypic forms are LTW-5A, found in strain B, and LTW-5B, found in strain C (Figures 1 and 6). Both spots are present in the heterozygote but with reduced intensity. The strain distribution pattern is similar to that for *Car-2* (carbonic anhydrase isozyme II), which maps

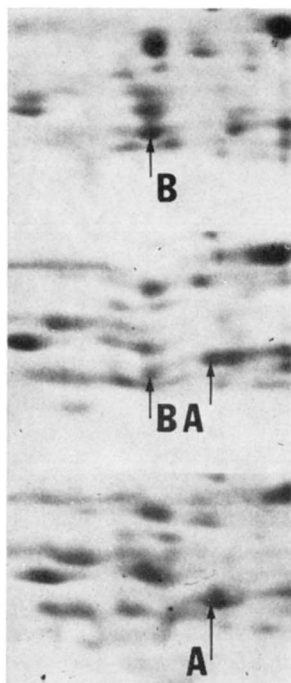


FIGURE 6.—Enlargement of part of two dimensional gel electrophoresis patterns showing LTW-5. The three panels are arranged as in Figure 3. Note the present of a spot (B) in the upper panel, which is absent in the lower panel. The spot (A), present in the lower panel, is absent in the upper panel. Spots in both positions are found in the F_1 heterozygote.

on Chromosome 3 (EICHER, *et al.* 1976) and to the histocompatibility locus, *H-28*, and NDV-induced circulating interferon, *If-1*. These latter two loci are linked to each other but not to *Car-2*. Their chromosome location is not yet known.

LTH-1: This variant spot, with molecular weight close to 35,000, is present in strain C and absent in strain B (Figures 1 and 7). The spot is faint and not easy to score unless the gel has a very low background staining. In the heterozygote, the spot is present, but even more faint. The strain distribution pattern for *Lth-1* differs by one strain from that of the histocompatibility genes *H-17*, *H-34* and *H-38*, so that no tentative map assignment can be made at this time.

LFO-1: The last variant in Table 1 is LFO-1. The LFO-1 spot is quite large, probably representing 1 to 2% of the supernatant protein, and more basic than the other variants. The more acidic phenotypic form, LFO-1A, is present in strain B, while the more basic, LFO-1B, is found in strain C (Figures 1 and 8). In the

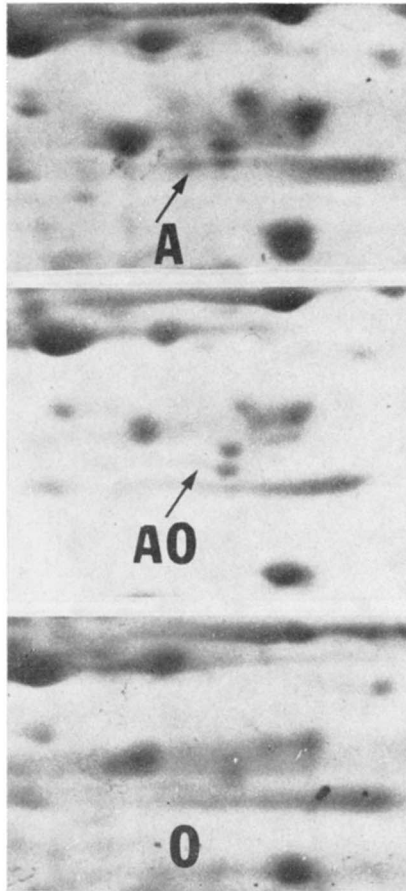


FIGURE 7.—Enlargement of part of two dimensional electrophoresis patterns showing LTW-1. The three panels are arranged as in Figure 3. Note that a spot marked (A) is found in the upper panel, but is missing in the lower panel. There is a light spot in this position in the F_1 heterozygote.

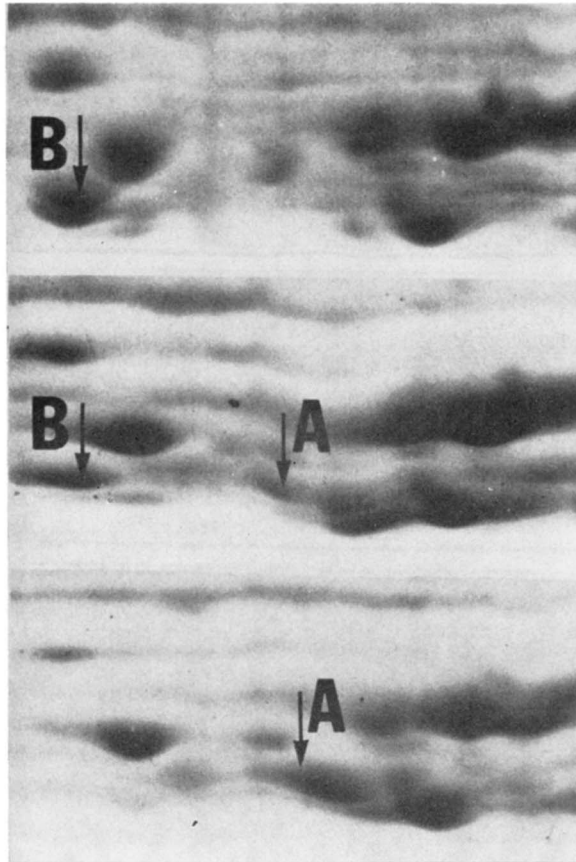


FIGURE 8.—Enlargement of part of two-dimensional electrophoresis patterns showing LFO-1. The three panels are arranged as in Figure 3. The basic spot marked (B) present in the upper panel is missing in the lower panel, while the more acidic spot marked (A) in the lower panel is missing in the upper panel. Both spots are present, but at lower intensity, in the panel from the F_1 heterozygote.

heterozygote, both spots are present but with decreased intensity. The strain distribution pattern for *Lfo-1* is similar to that for three genes on Chromosome 7, the albino locus, *c*, the histocompatibility locus, *H-1* (SNELL and STEVENS 1961), and the structural locus for the β chain of hemoglobin, *Hbb* (POPP and ST. AMAND 1960). Thus *Lfo-1* has been tentatively assigned to Chromosome 7.

Confirmation of chromosome assignments: Congenic resistant strains have been made for a number of histocompatibility loci scattered throughout the mouse genome (SNELL 1958; BAILEY 1975). In many cases the allele from BALB/6By has been backcrossed into the C57BL/6By background. In addition, the C \times B RI strains have been analyzed for these loci. As shown in Table 1, some of the histocompatibility loci have strain distribution patterns identical with those found in this study. Liver cytosols from appropriate congenic resistant strains have been studied using the two dimensional electrophoresis

TABLE 2

Phenotypes of eight polypeptides among congenic strains

Polypeptide	H-1	H-7	Phenotype* in congenic strains			H-35	H-38
			H-17	H-28	H-34		
LTN-1	B	B	B	B	B	B	B
LTW-1	B	B	B	B	B	B	B
LTW-2	B	B	B	B	B	B	B
LTW-3	B	C	B	B	B	B	B
LTW-4	B	B	B	B	B	B	B
LTW-5	B	B	B	B	B	B	B
LTH-1	B	B	B	B	B	B	B
LFO-1	C	B	B	B	B	B	B

* In order to facilitate comparisons, phenotypes are designated as to whether they are like that found in the majority parent, (B) or in the minority parent, (C).

techniques. Each of the variant polypeptide spots has been analyzed as to whether its phenotype is similar to that in strain B or in strain C. As shown in Table 2, most phenotypes are those of the majority parent, C57BL/6By. In some cases, however, the allele from strain BALB/cBy has survived the backcrossing, and is expressed in the congenic mouse. This would be expected for genes closely linked to the selected histocompatibility gene.

Using the congenic strains, confirmation of two of the chromosome assignments has been obtained. The assignment of gene *Ltw-3* to Chromosome 9 has been confirmed by the transfer of the *Ltw-3^a* allele from strain C, along with the selected *H-7^b* allele into the congenic strain, B6.C-*H-7^b*. Similarly, the assignment of gene *Lfo-1* has been confirmed by the transfer of the *Lfo-1^b* allele, with the selected *H-1^b* allele, into the congenic strain B6.C-*H-1^b*. No conclusion can be reached about the map positions of the other loci in this study, as there is no way to distinguish between absence of linkage and exclusion of a linked marker from the region of donor chromosome transferred to the recipient in the generation of a congenic resistant strain.

DISCUSSION

This is the first genetic study of the major soluble proteins of mouse liver cytosol. In comparing the two strains C57BL/6By and BALB/cBy, a majority of the 200-250 distinct spots were superimposable when the samples were run in the same experiment. Most of the spots were in two molecular weight classes, approximately 25,000 and 45,000. About 5% of the spots differed consistently between the two strains. When F₁ animals were studied, the spot pattern was as expected for genetically determined, co-dominantly expressed polypeptides.

The initial attempt to map the genes coding for the polypeptides was done using recombinant inbred lines. As suggested by BAILEY (1971), the R1 strains afford a number of advantages over performing crosses and studying individual progeny. (1) All animals of a strain have the same genotype and a number of animals from the same strain may be studied. (2) There are no heterozygote

phenotypes, so that the classification of genetic differences is more straightforward. (3) Data on the strains are cumulative. The strain distribution patterns for all loci previously analyzed in the strains are continuously valid, and the strains do not have to be reanalyzed for these loci, whereas in a cross each animal has to be analyzed for all loci. (4) The time delay required to produce sufficient progeny animals in a cross is eliminated.

The RI strains between strains C57BL/6By and BALB/cBy have now been analyzed for over 30 markers. The probability of finding a matching strain distribution pattern is therefore very high. The total number of strain distribution patterns for seven lines is 128. Although the finding of identical strain distribution patterns does not prove linkage, the suggested linkage could be most easily checked if appropriate congenic strains were available.

Of the eight variant spots that appeared to be genetically determined, tentative chromosome assignments have been made for five. Two others may be linked to previously unmapped histocompatibility loci, and one shows no evidence of linkage to any known loci.

The identity of the native proteins associated with most of the polypeptide spots on the gel so far is unknown. The variant LTN-1, a set of three spots, is most probably MUP. A second variant, LTW-1, is a plasma polypeptide whose identity has not yet been established. Although the identity of the spots should be determined eventually, the lack of knowledge of the function of the protein product does not limit the genetic studies. The approximate molecular weights and isoelectric points of the polypeptides are determined in the study.

Two-dimensional gels of liver cytosols have been studied for a number of mouse strains (unpublished results). Among the seven strains tested so far, fourteen electrophoretic variants have been found. Comparison of any pair of mouse strains will allow study of four to ten of these differences. This suggests that analysis of any set of RI strains by two-dimensional electrophoresis will yield as much genetic information as the analysis of the C × B RI strains reported here. Other sets of RI strains, which contain more than seven inbred strains derived from two progenitor strains, may in fact be even more useful genetically. The first case of linkage detection, that for the *ald* (adrenal lipid depletion) locus, was reported by TAYLOR and MEIER (1976), who used a set of 21 RI strains derived from the progenitor strains AKR/J and C57L/J.

Once the genes controlling the synthesis of the eight polypeptide spots are mapped, these loci should become very useful in mapping other new and physiologically important genes. A mouse from a RI strain or a progeny mouse from a cross can now be analyzed for eight phenotypes simultaneously, using a single technique. As markers from other organs or from membrane proteins are added, the fraction of the genome covered will increase rapidly. When other electrophoretic markers are added, it may be possible to survey the total mouse genome in a cross between any pair of strains.

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